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ATTORNEY'S DOCKET NUMBER
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**TRANSMITTAL LETTER TO THE
UNITED STATES
DESIGNATED/ELECTED OFFICE
(DO/EO/US) CONCERNING A FILING
UNDER 35 U.S.C. 371**

U.S. APPLICATION NO.
(if known, sec 37 C.F.R.1.5)

09/673412

INTERNATIONAL APPLICATION NO.
PCT/IL99/00217INTERNATIONAL FILING DATE
April 23, 1999PRIORITY DATE CLAIMED
April 27, 1998TITLE OF INVENTION
PHARMACEUTICAL COMPOSITION COMPRISING FACTOR VIII AND NEUTRAL LIPOSOMESAPPLICANTS FOR DO/EO/US
Moshe BARU, Liliana BAR, Israel NUR

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
 - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☒ has been transmitted by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☐ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☐ A FIRST preliminary amendment.
 - ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ Entitlement to small entity status is hereby asserted.
16. ☒ Other items or information: PCT Request

U.S. APPLICATION NO. (if known, see 37 C.F.R. 1.5) 09/673412	INTERNATIONAL APPLICATION NO. PCT/IL99/00217	ATTORNEY'S DOCKET NUMBER 107587
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17. <input checked="" type="checkbox"/> The following fees are submitted: Basic National fee (37 CFR 1.492(a)(1)-(5)): Search Report has been prepared by the EPO or JPO\$860.00 International preliminary examination fee paid to USPTO (37 CFR1.482)\$690.00 No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2))\$710.00 Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO\$1,000.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4)\$ 100.00 ENTER APPROPRIATE BASIC FEE AMOUNT = \$860.00	CALCULATIONS PTO USE ONLY																								
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	\$																								
<table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 20%;">Claims</th> <th style="width: 20%;">Number Filed</th> <th style="width: 10%;">Number Extra</th> <th style="width: 10%;">Rate</th> <th style="width: 10%;"></th> <th style="width: 10%;"></th> </tr> <tr> <td>Total Claims</td> <td>19- 20 =</td> <td>0</td> <td>X \$ 18.00</td> <td>\$</td> <td></td> </tr> <tr> <td>Independent Claims</td> <td>5- 3 =</td> <td>2</td> <td>X \$ 80.00</td> <td>\$80.00</td> <td></td> </tr> <tr> <td colspan="3">Multiple dependent claim(s) (if applicable)</td> <td>+ \$270.00</td> <td>\$</td> <td></td> </tr> </table>	Claims	Number Filed	Number Extra	Rate			Total Claims	19- 20 =	0	X \$ 18.00	\$		Independent Claims	5- 3 =	2	X \$ 80.00	\$80.00		Multiple dependent claim(s) (if applicable)			+ \$270.00	\$		
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a. <input checked="" type="checkbox"/> Check No. 112869 in the amount of \$940.00 to cover the above fees is enclosed. b. <input type="checkbox"/> Please charge my Deposit Account No. _____ in the amount of \$_____ to cover the above fees. A duplicate copy of this sheet is enclosed. c. <input checked="" type="checkbox"/> The Director is hereby authorized to charge any additional fees which may be required, or credit any overpayment, to Deposit Account No. 15-0461. A duplicate copy of this sheet is enclosed.	<div style="text-align: center;"> NAME: James A. Oliff REGISTRATION NUMBER: 27,075 NAME: Thomas J. Pardini REGISTRATION NUMBER: 30,411 </div>
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NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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VARTS

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PHARMACEUTICAL COMPOSITION COMPRISING FACTOR VIII AND NEUTRAL LIPOSOMES

FIELD OF THE INVENTION

The present invention relates to a stable pharmaceutical formulation for the slow release of coagulation promoting substances for the treatment of blood coagulation disorders.

BACKGROUND OF THE INVENTION

Hemophilia A is one of the most frequently occurring inherited coagulation disorders. Patients with hemophilia A are prone to frequent hemorrhages as a result of one or more misfunctions of the coagulation system. One of the causes of hemophilia is a shortage of Factor VIII (FVIII) in the blood. This problem can be treated with Factor VIII concentrates. However, in about 15% of the patients the occurrence results of Factor VIII neutralizing antibodies, so-called inhibitors, whereby a therapy with Factor VIII concentrates is hardly possible.

Two basic approaches have been described in the literature to protect FVIII from inactivation by inhibitors.

WO/80/01456 to Hemker discloses a pharmaceutical composition suitable for oral administration comprising FVIII incorporated within liposomes of 0.5-1.0 microns formed from phospholipids. The phospholipids have a net charge, and the FVIII is incorporated between the

layers of the liposome. It is claimed that FVIII levels in the plasma remained above about 5% of the normal value for a period of 50 hours.

US 4,348,384 to Horikoshi states that a composition as described in Hemker was prepared, but did not give satisfactory results.

- 5 Therefore, Horikoshi incorporates a protease inhibitor into the liposome together with FVIII, in order to protect it from proteolysis. 3% of the normal plasma levels of FVIII were obtained over a period of 6 hours.

- US 5,013,556 to Woodle discloses a liposome composition for use in delivering various drugs via the bloodstream. The liposome contains
10 between 1-20 mole percent of an amphipathic lipid derivatized with a polyalkylether. Here also, the drug compound is entrapped within the liposome. These liposome compositions are available commercially under the name of Stealth® vesicles (SUV's, small unilamellar vesicles comprised of phospholipid and polyethylene glycol (PEG) covalently bound to
15 phospholipid).

A further problem with this approach is that liposomes having a large diameter have a short half-life. Therefore, the liposomes must be downsized under high pressure, which can affect protein activities as in coagulation factors V and VIII.

- 20 In a second approach, Barrowcliffe, T.W., *et al.* (1983) J. Lab. Clin. Med. 101:34-43 teaches that mixing FVIII with phospholipid extracted from human and/or animal brain imparts significant protection to the FVIII *in vitro*. In this approach, the phospholipid is bound to the FVIII rather than encapsulating it. Kemball-Cook, G. and Barrowcliffe, T.W. (1992) Thromb.
25 Res. 67:57-71, teaches that a negatively-charged phospholipid surface is necessary for FVIII binding. Negatively charged phosphatidyl serine and phosphatidic acid were found to be highly active in binding to FVIII, while phosphatidyl choline was inactive. However, negatively-charged

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phospholipids are toxic, and those derived from brain tissue may carry pathogenic agents.

EP 689,428 discloses a liposome composition comprising liposomes having an outer surface layer of hydrophilic polymer chains. A polypeptide or polysaccharide effector molecule is covalently attached to the distal ends of the polymer chains by activation of the lipid anchor prior to effector coupling.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide a pharmaceutical composition comprising a protein or polypeptide for therapeutic treatment. In particular, it is an object of the present invention to provide a pharmaceutical composition comprising FVIII for the treatment of blood coagulation disorders.

It is a further object of the invention to provide FVIII in a form having an extended half-life in the bloodstream.

It is a still further object of the invention to provide a method for treating patients suffering from blood coagulation disorders, particularly hemophilia, and most particularly those having FVIII inhibitors.

In one aspect of the present invention there is provided a pharmaceutical composition for parenteral administration comprising a therapeutically effective amount of coagulation factor VIII (FVIII) and substantially neutral colloidal particles, the particles comprising 1-20 mole percent of an amphipathic lipid derivatized with a biocompatible hydrophilic polymer, the polymer carrying substantially no net charge, wherein the FVIII is not encapsulated in the colloidal particles.

The present invention is based on the surprising and unexpected finding that neutral phospholipids derivatized with a bio-compatible hydrophilic polymer can be used to bind FVIII and protect it from inhibitors

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in the bloodstream. This provides a significant advantage over the prior art compositions, since the phospholipids used are synthetic and non-toxic, and can therefore be used *in vivo* for therapeutic treatment. Furthermore, the liposome does not encapsulate the FVIII so that smaller sized liposomes can
5 be used which have a longer half-life *in vivo*, since they are not removed by the reticuloendothelial system (RES). As will be described below in greater detail, FVIII interacts non-covalently with the polymer chains on the external surface of the liposomes, and no chemical reaction is carried out to activate the polymer chains, unlike the composition disclosed in EP 689,428.

10 In the present specification, the terms "*substantially neutral*" and "*substantially no net charge*" mean neither positively nor negatively charged. However, a very low measured charge within experimental error of zero is included within the meaning of the above terms.

The term "*therapeutically effective amount*" is to be understood
15 as referring to an amount of FVIII which results in a level of FVIII in the bloodstream having a desired therapeutic effect. Such an amount can be experimentally determined by administering compositions comprising different amounts of FVIII and measuring the level in the blood at various times after administration.

20 The amphipathic lipid used to prepare the colloidal particles is preferably a phospholipid, and may be obtained from either natural or synthetic sources. A most preferred phospholipid is phosphatidylcholine, most preferably egg-phosphatidylcholine.

The biocompatible hydrophilic polymer may include polymers
25 from the polyalkylether, polylactic or polyglycolic acid families. Preferably, the polymer is polyethylene glycol (PEG). The purpose of the polymer is to sterically stabilize the SUVs, thus preventing fusion of the vesicles *in vitro*, and allowing the vesicles to escape adsorption by the RES *in vivo*. The

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polymer will preferably have a molecular weight of between about 1000 to about 5000 daltons, most preferably approximately 2000 daltons.

The colloidal particles will preferably have a mean particle diameter of between about 0.05 to about 0.4 microns, most preferably about 0.1 microns. This is to increase their circulation time *in vivo* and prevent their adsorption by the RES. The amphipathic lipid comprises approximately 1 to about 20 mole % of the particles, preferably approximately 1-5%, most preferably 5%.

A variety of known coupling reactions may be used for preparing vesicle forming lipids derivatized with hydrophilic polymers. For example, a polymer (such as PEG) may be derivatized to a lipid such as phosphatidylethanolamine (PE) through a cyanuric chloride group. Alternatively, a capped PEG may be activated with a carbonyl diimidazole coupling reagent, to form an activated imidazole compound. Other reactions are well known and are listed, e.g. in the aforementioned U.S. 5,013,556, whose contents are incorporated herein by reference.

The FVIII used in the composition of the invention is commercially available. It may be from a natural human source, or, preferably, it may be recombinantly prepared. Recombinant FVIII is commercially available, for example, Antihemophilic Factor (Recombinant), rFVIII-SQ (Pharmacia), and Kogenate, Miles Inc., Pharmaceutical Division, Elkhart, IN, U.S.A., among other suppliers.

The composition of the invention is administered parenterally, preferably iv. The prior art compositions were intended for oral use only, due to side effects caused during injection by the liposome composition. The composition of the invention, on the other hand, is not toxic by injection, apparently due to the lack of charge, among other causes. Amounts of up to 0.5gm/Kg body weight of colloidal particles according to the invention have been injected without detectable toxic symptoms. The dose is expected to be

in the approximate range of 25-75 i.u./Kg. body weight. The particle to FVIII ratio (w/unit FVIII) will preferably be between about 0.1 mg/unit and about 10 mg/unit, and most preferably, approximately 1 mg/unit. Although the free form of FVIII:C has a half-life of less than 2 hours (FVIII measured by clotting activity) in mice, FVIII administered in the composition of the invention is expected to be effective for at least 24 hours, which is the period of effective activity of the coagulation promoting compound. The composition of the invention is expected to be effective in "on demand" and prophylactic treatment of hemophilia patients, and particularly those patients who have developed FVIII inhibitor antibodies.

The effectiveness of FVIII contained in the composition of the invention may be determined by a chromogenic assay which determines FVIII activity by two consecutive steps: (1) the FVIII-dependent conversion of Factor X to Factor Xa in a coagulation-factor reagent composed of purified components, and (2) the enzymatic cleavage of a chromogenic Factor Xa substrate to yield a chromophore which can be quantified spectrophotometrically. Under appropriate assay conditions, there exists a linear relationship between the rate of Factor Xa formation and the FVIII concentration. In addition, FVIII activity may be determined by a one-stage clotting assay. This assay determines FVIII activity by the conversion of prothrombin to thrombin, which subsequently cleaves fibrinogen to form a clot composed of fibrin. FVIII activity in hemophilic mice may also be determined by measuring the survival of the mice following a tail cut.

In a further aspect of the invention, there is provided a pharmaceutical composition for parenteral administration comprising a therapeutically effective amount of a protein or polypeptide and substantially neutral colloidal particles, said particles comprising approximately 1-20 mole percent of an amphipathic lipid derivatized with a biocompatible hydrophilic polymer, said polymer carrying substantially no net charge, wherein said

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protein or polypeptide is selected from the group consisting of: (a) proteins or polypeptides capable of externally binding said colloidal particles; and (b) proteins of polypeptides capable of binding polyethylene glycol (PEG), and wherein said protein or polypeptide is not encapsulated in said colloidal particles.

The term "*proteins or polypeptides capable of externally binding said colloidal particles*" includes proteins and polypeptides which, similarly to FVIII, bind to membranes comprising phosphatidylcholine: phosphatidylserine (PC:PS) (see Haemostasis and Thrombosis. Arthur L. Bloom and Duncan P. Thomas (eds) (1987) Churchill Livingstone, pg. 179-180). Non-limiting examples of such proteins are coagulation factors such as prothrombin, Factor X and Factor V.

The term "*proteins or polypeptides capable of binding polyethylene glycol*" includes proteins and polypeptides which bind to PEG or derivatives of PEG by any non-covalent mechanism, such as ionic interactions, hydrophobic interactions, hydrogen bonds and Van der Waals attractions (Arakawa, T. and Timasheff, S.N. (1985) Biochemistry 24:6756-6762; Lee, J.C. and Lee, L.L.Y. (1981) J. Biol. Chem. 226:625-631).

BRIEF DESCRIPTION OF THE DRAWING:

In order to understand the invention and to see how it may be carried out in practice, preferred embodiments will now be described, by way of non-limiting example only, with reference to the accompanying drawing which illustrates survival of hemophilic mice injected with FVIII following a tail cut at various time periods post-injection.

DETAILED DESCRIPTION OF A PREFERRED EMBODIMENT

Examples 1-3

Methods and Materials

1. Egg phosphatidylcholine (E-PC) liposomes

5 A tert-butanol solution of egg phosphatidylcholine (E- PC) was prepared by dissolving 2.0 gr. E-PC, 1.9 mg α -tocopherol and fluorescein-labeled phosphatidylethanolamine (1:1000 lipid molar ratio) in 18ml tert-butanol.

The organic solvent was removed from the lipidic mixture by
10 lyophilization and the lipids reconstituted in water to 10 % w/v. The obtained liposomes were reduced in size by extruding them through a series of polycarbonate (PC) filters (0.4 μ m, 0.2 μ m, 0.1 μ m and 0.05 μ m) using the Liposofast-Basic or Liposofast-50 extruder (Avestin) to obtain liposomes of an average size of 0.1 μ m.

15

2. Egg phosphatidylcholine/polyethyleneglycol-phosphatidyl ethanolamine (E-PC/PEG-PE) liposomes

A tert-butanol solution of egg phosphatidylcholine (E- PC) and polyethyleneglycol-phosphatidyl ethanolamine (PEG-PE) was prepared by
20 mixing 0.73 gr. E-PC, 0.185 gr. PEG-PE, 0.86 mg α -tocopherol and fluorescein labeled phosphatidylethanolamine (1:1000 lipid molar ratio) in 18ml tert-butanol.

The organic solvent was removed from the lipidic mixture by
lyophilization and then reconstituted in water to 10 % w/v. The obtained
25 liposomes were reduced in size as described in 1 above to obtain liposomes of an average size of 0.1 μ m.

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3. Egg phosphatidylcholine-phosphatidyl glycerol (E-PC/PG) liposomes

A tert-butanol solution of egg phosphatidylcholine (E- PC) and phosphatidyl glycerol (PG) was prepared by mixing 0.822 gr. E-PC, 0.0924
5 gr. PG, 0.86 mg α -tocopherol and phosphatidylethanolamine fluorescein labeled (1:1000 lipid molar ratio) in 18ml tert-butanol.

The organic solvent was removed from the lipidic mixture by lyophilization and then reconstituted in water to 10 % w/v. The obtained liposomes were reduced in size as described in 1 above to obtain liposomes of
10 an average size of 0.1 μ m.

4. Reconstitution of the human recombinant factor VIII:

Kogenate (rFVIII formulated with human albumin, Bayer) lots 70K026 and 70K027, were used in the following examples. One vial
15 containing about 500 IU of FVIII activity was reconstituted with 2 ml water and allowed to solubilize. 200 μ l aliquots were frozen at -20°C until use.

For the preparation of albumin-depleted Kogenate, lot # 70K027 was used. 10 vials of Kogenate were reconstituted in 20 ml water and chromatographed on a hydrophilic silica gel (3-10 μ m beads). Fractions of
20 10ml were collected and the protein and FVIII:Ag activities were monitored.

A 50 % recovery in FVIII:Ag activity was found in one peak of fractions 4-6 and another of fractions 8-14. Since the protein assay gave a peak at fractions 9-12, fractions 4-6 were pooled, aliquoted and lyophilized for further use.

- 25 5. Hemophilic mice prepared as described in Bi, L., *et al.* (1995) Nature Genetics 10:119-121, were used.

6. FVIII:Ag activity was determined using a FVIII chromogenic assay commercially available from Dade AG, Dudingon, Switzerland.

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7. Preparation of composition and injection to hemophiliac mice
A liposomal aliquot was mixed with a predetermined volume of FVIII to obtain a FVIII:Ag activity of 5-10 IU/ml and rolled at RT to achieve
5 homogeneity.
8. Groups of 5-10 hemophiliac mice were injected IV bolus through the tail vein, with 200 or 400 μ l of the mixture. The mice were bled from the eye at regular time intervals (1h, 4hs, 8hs, 24hs, 32hs and 48hs) and
10 the FVIII:Ag activities in the plasma were followed.
9. The pharmacokinetics of FVIII was determined from the results by using the RSTRIP computation software to obtain the initial FVIII:C activity (A_0) and the half-life time ($T_{1/2}$) of the factor in the mice blood
15 circulation.

Results

1. Effect of Lipid composition on the Half-Life of Factor VIII.
Liposomes of 0.05 μ m comprising E-PC, E-PC/PEG-PE and
20 E-PC/PG were prepared, mixed with Kogenate in a 72:1 lipid to protein (w/w) ratio and injected into hemophiliac mice. As a control, Kogenate was diluted in saline and injected into the mice in the same manner as the liposomal mixtures. The pharmacokinetic parameters were determined as described above, and the results are summarized in the following table:

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Table #1: Effect of lipid composition on the half-life of FVIII

Lipid composition	A ₀ * (IU/ml)	T _{1/2} (hs)	no. of mice
Control	2.22	4.51	18
E-PC/PEG-PE	3.20	7.84	10
E-PC	1.01	2.33	10
E-PC/PG	Not-detectable	not-detectable	10

* A₀ = initial concentration of FVIII:C

It can be seen from the table that liposomes containing E-PC /PEG-PE were the most effective since both the initial FVIII activity and the half-life time were higher for this composition than for Kogenate or Kogenate-liposome mixtures where the liposomes were composed of E-PC/PG or E-PC only.

Moreover, 40% of the mice injected with free FVIII and 100% of the mice injected with FVIII /PC complex did not exhibit any recovery of FVIII chromogenic activity, while only 10% of the mice injected with FVIII/PC+PEG exhibited the same phenomena 60 min. after injection.

2. Effect of Lipid/Protein Ratio on the Half-Life of Factor VIII.

Various lipid to protein ratios in the liposome composition were obtained by mixing various aliquots of liposomes of 0.05μm comprising E-PC/PEG-PE with Kogenate. These were injected into hemophilic mice. As a control, Kogenate was diluted in saline and injected into the mice in the same manner as the liposomal mixtures. The pharmacokinetic parameters were determined as described above, and the results are summarized in the following table:

Table #2: Effect of lipid to protein ratio on the half-life of FVIII

lipid/prot. (w/w)	A ₀ (IU/ml)	T _{1/2} (hs)	no. of mice
134	2.26	3.3	10
32	1.61	1.91	10
5.3	3.12	1.64	10
0.89	2.69	1.5	10
Control	2.22	1.5	18

It can be seen from Table #2 that increasing the lipid/protein ratio increases the half-life time of FVIII in the blood circulation in the hemophiliac mice. The differences in the initial FVIII:C activities appear not to be related to the lipid/protein ratio.

3. Effect of different Factor VIII sources

SUVs of 0.05 μ m were prepared containing E-PC and PEG-PE (94:6 mol %), mixed with FVIII concentrates from various sources (Kogenate, Baxter and Omrixate) in a 72:1 lipid to protein ratio and injected into hemophiliac mice. As a control, each FVIII concentrate from the various sources was diluted in saline and injected into the mice in the same manner as the liposomal mixtures. The pharmacokinetic parameters were determined as described above, and the results are summarized in the following table:

Table #3: Effect of factor FVIII source on the half-life of FVIII

source	A ₀ (IU/ml)	T _{1/2} (hs)
Kogenate	2.22	4.51
Kogenate+ SUV's	3.36	8.60
Baxter	1.36	3.83
Baxter+ SUV's	1.08	4.45
Omrixate	2.35	3.21
Omrixate + SUV's	2.31	3.90

- Mixtures containing liposomes and FVIII from Baxter or
- 5 Omrixate increased the half-life of the factor by 20%, when compared with the pharmacokinetic values of the free factor, as can be seen from the above table. The half-life of factor FVIII from Kogenate, mixed with E-PC/PEG-PE liposomes was twice as long as compared with the free factor form.

10 **Example 4**

Methods and materials

1. Liposome preparation:

- Liposomes were prepared as follows: Egg phosphatidyl choline (EPC) and distearoyl phosphatidyl-ethanolamine methyl polyethylene glycol
- 15 2000 (DSPE-PEG 2000) were weighed to a ratio of 80:20 w/w (5% molar ratio of DSPE-PEG 2000), respectively, dissolved to 10% w/v in tert-butanol (Reidel-de Haen), and the solution was lyophilized. The obtained dry lipid powder was resuspended to 10% w/v in a buffer containing 130 mM NaCl, 10 mM sodium citrate, 1 mM CaCl₂ pH 7.0 to form liposomes. The liposomes

were filtered in an extruder apparatus (Avestin) through polycarbonate filters 1.2 μm , 0.2 μm and 0.1 μm in size to form liposomes of 120-140 nm in size.

2. Liposome quality control

5 Quality control of the liposomes included:

- 1) Size distribution measured by sub-micron particle analyzer (N4 plus, Coulter Electronics).
- 2) Phospholipid determination (by phosphorus).
- 3) Chemical stability of the lipids by TLC.

10 The tests were performed as described in: Barenholz, Y. and Amselem, S. (1993) in Liposome Technology, 2nd edition, Vol. I (Gregoriadis, G., ed.), CRC Press, Boca Rayton, FL, pp.527-616.

3. Formulation of FVIII and liposomes

15 Kogenate (Lot no. 70K027, 620 IU) or New Kogenate (500 IU) was dissolved in 1 ml or 2 ml of H_2O . The rFVIII-SQ concentrate was dissolved in liposome solution. Factor VIII was formulated with liposomes by mixing FVIII concentrate with the liposomes for about 1 hour at room temperature. The ratio of lipids to FVIII units was about 1 mg lipids/1 unit
20 FVIII.

4. Injection into hemophilic mice and bleeding procedures

Factor VIII and FVIII formulated with liposomes were injected into the tail vein of hemophilic mice. The injected dose was 3 units/mouse for
25 Kogenate (2 separate experiments) and New Kogenate and 4 units/mouse for rFVIII-SQ. The mice were bled into citrate tubes at 10 minutes after the injection and at about 4, 19 and 27 hours post-injection.

5. Measurement of FVIII concentrate in mouse plasma

Human FVIII concentrate in mouse plasma was measured using a chromogenic assay (Chromogenix) according to the manufacturer's instructions, and by one stage clotting assay (using Stago reagents and ST4 clotting machine) according to the manufacturer's instructions.

6. Pharmacokinetics analysis

Pharmacokinetics parameters were analyzed using a computer program (RSTRIP, MicroMath Inc.).

7. Survival of hemophilic mice following a tail cut

Mice were injected with free rFVIII-SQ or liposome formulated rFVIII-SQ (4 units/mouse, 11 mice in each group. At 20 hours post injection 2 cm of the tail were cut. Tails of the surviving mice were cut again at 28, 44, 52, 69, 88 and 140 hours post-injection (2 mm each time).

Results

The results of FVIII activity at each time point post-injection and the pharmacokinetic parameters [FVIII half-life (HL) and the area under the curve (AUC)] of 4 different experiments are summarized in Tables 4-7B. In Tables 4 and 5, 3 units of Kogenate /mouse were injected; In Table 6, 3 units of New Kogenate /mouse were injected; and in Tables 7A, 7B, and in Fig. 1, 4 units of rFVIII-SQ /mouse were injected.

Table 4: Factor VIII activity (u/ml measured by a chromogenic assay) and pharmacokinetic parameters following injection of human FVIII into hemophilic mice

Injected Material		T = 10 min.	T = 4.5 hours	T = 19 hours	T = 27 hours	Area under the curve (AUC) (IU*h/ml)	Half life (HL) (h)
Kogenate (n=7)	Average (u/ml) \pm SD	2.878 \pm 0.571	0.450 \pm 0.392	0.015 \pm 0.0043	0.0016 \pm 0.0004	7.218	1.619
Injected Material		T = 10 min.	T = 3.6 hours	T = 18.1 hours	T = 26.1 hours		
Kogenate + liposomes (n=7)	Average (u/ml) \pm SD	2.951 \pm 0.333	1.121 \pm 0.337	0.023 \pm 0.003	0.014 \pm 0.0018	10.969	2.460

Table 5: Factor VIII activity (u/ml measured by a chromogenic assay) and pharmacokinetic parameters following injection of human FVIII into hemophilic mice

Injected Material		T = 10 min.	T = 3.334 hours	T = 19.334 hours	T = 26.3 hours	Area under the curve (AUC) (IU*h/ml)	Half life (HL) (h)
Kogenate (n=8)	Average (u/ml) \pm SD	3.686 \pm 0.674	0.960 \pm 0.469	0.0128 \pm 0.007	0.0025 \pm 0.006	9.314	1.632
Injected Material		T = 10 min.	T = 3.5 hours	T = 19.5 hours	T = 26.5 hours		
Kogenate + liposomes (n=8)	Average (u/ml) \pm SD	3.618 \pm 0.982	1.571 \pm 0.137	0.032 \pm 0.009	0.012 \pm 0.009	15.059	2.771

Table 6: Factor VIII activity (u/ml measured by a chromogenic assay) and pharmacokinetic parameters following injection of human FVIII into hemophilic mice

Injected Material		T = 10 min.	T = 2.5 hours	T = 17 hours	T = 26 hours	Area under the curve (AUC) (IU*h/ml)	Half life (HL) (h)
New Kogenate (n=4)	Average (u/ml) \pm SD	1.841 \pm 0.643	0.120 \pm 0.036	0.004 \pm 0.003	0.0015 \pm 0.003	1.910	0.592
Injected Material		T = 10 min.	T = 2.666 hours	T = 17.166 hours	T = 26.166 hours		
New Kogenate + liposomes (n=6)	Average (u/ml) \pm SD	2.393 \pm 0.243	0.352 \pm 0.131	0.011 \pm 0.0019	0.008 \pm 0.001	3.544	0.904

Table 7A: Factor VIII activity (u/ml measured by a chromogenic assay) and pharmacokinetic parameters following injection of human FVIII into hemophilic mice

Injected Material		T = 10 min.	T = 4.166 hours	T= 20.166 hours	Area under the curve (AUC) (IU*h/ml)	Half life (HL) (h)
RVIII (n=10)	Average (u/ml) \pm SD	3.937 \pm 0.449	0.444 \pm 0.131	0	7.9	1.270
Injected Material		T = 10 min.	T = 4.5 hours	T= 20.5 hours		
RVIII + liposome s (n=11)	Average (u/ml) \pm SD	3.828 \pm 1.08	0.555 \pm 0.198	0.005 \pm 0.008	9.249	1.555

Table 7B: Factor VIII activity (u/ml measured by a one stage clotting assay) and pharmacokinetic parameters following injection of human FVIII into hemophilic mice

Injected Material		T = 10 min.	T = 4.166 hours	T = 20.166 hours	Area under the curve (AUC) (IU*h/ml)	Half life (HL) (h)
RVIII (n=9)	Average (u/ml) \pm SD	4.181 \pm 1.275	1.742 \pm 0.778	0.414 \pm 0.085	21.246	3.521
Injected Material		T = 10 min.	T = 4.5 hours	T = 20.5 hours		
RVIII + liposome s (n=10)	Average (u/ml) \pm SD	3.305 \pm 0.831	2 \pm 0.709	0.531 \pm 0.147	28.718	6.900

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In addition, the half-life of human FVIII in each mouse was calculated and the FVIII half-lives in all the experimental groups were statistically compared to each other by a student t-test.

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The statistical analysis indicates that in all 4 experiments human FVIII half-lives in the groups that received liposome-formulated FVIII were higher and significantly different ($p < 0.055$) from human FVIII half-lives in the groups that received free FVIII:

- HL of Kogenate versus HL of Kogenate + liposomes (table 4) $p=0.054$;
HL of Kogenate versus AUC of Kogenate + liposomes (table 5) $p=0.031$;
HL of New Kogenate versus HL of New Kogenate + liposomes (table 6)
 $p=0.0085$;
5 HL of rFVIII-SQ versus HL of rFVIII-SQ + liposomes (table 7A)
 $p=0.0045$; HL of rFVIII-SQ versus HL of rFVIII-SQ + liposomes (table
7B) $p=0.022$).

These results indicate that the formulation of Kogenate, New
Kogenate or rFVIII-SQ with liposomes significantly increases the factor
10 half-life (HL) and the area under the curve (AUC) of FVIII in hemophilic
mice (factor of 1.6-2.0 for HL and AUC).

Survival of the mice is illustrated in Fig. 1. The results of the
tail cut experiment indicate that the liposome formulated FVIII is biologically
active longer than free FVIII, and therefore can protect hemophilic patients
15 for a longer period of time.

Example 5

Effectiveness of FVIII composition in patients with inhibitors

15 units of FVIII (Kogenate) were incubated for one hour at
room temperature with 120 nm liposomes (15 mg lipids) containing
20 EPC:DSPE-PEG2000 (95:5 mole %). Then, 1 unit of free FVIII (Kogenate)
or 1 unit of liposome formulated FVIII were incubated for 2 hours at 37°C
with various dilutions of a serum from a hemophilia patient who had
developed inhibitors (anti FVIII antibodies). After the incubation, the activity
of factor VIII was measured by a chromogenic assay.

25

The results are summarized in table 8:

Table 8: Activity (units/ml) of factor VIII in the presence of FVIII inhibitors

Serum dilution	Free FVIII	FVIII-liposomes
None	0	0.028
1:5	0.052	0.094
1:10	0.137	0.162
1:25	0.6	0.98
1:100	8.736	14.94

- 5 It can clearly be seen from this experiment that administration of FVIII together with the colloidal particles is effective in protecting the FVIII from serum inhibitors.

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CLAIMS:

1. A pharmaceutical composition for parenteral administration comprising a therapeutically effective amount of coagulation factor VIII (FVIII) and substantially neutral colloidal particles, said particles comprising
5 approximately 1-20 mole percent of an amphipathic lipid derivatized with a biocompatible hydrophilic polymer, said polymer carrying substantially no net charge,
wherein said FVIII is not encapsulated in said colloidal particles.
- 10 2. The pharmaceutical composition of Claim 1 wherein the colloidal particle has a mean particle diameter of between about 0.05 to about 0.4 microns.
3. The pharmaceutical composition of Claim 2 wherein the colloidal particle has a mean particle diameter of approximately 0.1 microns.
- 15 4. The pharmaceutical composition of Claim 1 wherein said amphipathic lipid is a phospholipid from natural or synthetic sources.
5. The pharmaceutical composition of Claim 4 wherein said amphipathic lipid is egg-phosphatidylcholine.
6. The pharmaceutical composition of Claim 1 wherein said
20 biocompatible hydrophilic polymer is selected from the group consisting of polyalkylether, polylactic and polyglycolic acid families.
7. The pharmaceutical composition of Claim 6 wherein said biocompatible hydrophilic polymer is polyethylene glycol.
8. The pharmaceutical composition of Claim 7 wherein the
25 polyethylene glycol has a molecular weight of between about 1000 to about 5000 daltons.
9. The pharmaceutical composition of Claim 8 wherein the polyethylene glycol has a molecular weight of approximately 2000 daltons.

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10. The pharmaceutical composition of Claim 1 wherein the FVIII is from a natural source.
11. The pharmaceutical composition of Claim 1 wherein the FVIII is recombinantly prepared.
- 5 12. The pharmaceutical composition of Claim 1 wherein the particle to FVIII ratio (w/unit FVIII) is between about 0.1 mg/unit and about 10 mg/unit.
13. The pharmaceutical composition of Claim 12 wherein the particle to FVIII ratio (w/unit FVIII) is approximately 1 mg/unit.
- 10 14. Method of treatment of a patient suffering from hemophilia comprising administering to said patient a pharmaceutical composition for parenteral administration comprising a therapeutically effective amount of coagulation factor VIII (FVIII) and substantially neutral colloidal particles, said particles comprising approximately 1-20 mole percent of an amphipathic
- 15 lipid derivatized with a biocompatible hydrophilic polymer, said polymer carrying substantially no net charge,
- wherein said FVIII is not encapsulated in said colloidal particles.
15. A method according to Claim 14 wherein said patient has
- 20 developed FVIII inhibitor antibodies.
16. Use of a colloidal particle in the preparation of a pharmaceutical composition for parenteral administration for treatment of a patient suffering from hemophilia comprising a therapeutically effective amount of coagulation factor VIII (FVIII) and substantially neutral
- 25 colloidal particles, said particles comprising approximately 1-20 mole percent of an amphipathic lipid derivatized with a biocompatible hydrophilic polymer, said polymer carrying substantially no net charge,
- wherein said FVIII is not encapsulated in said colloidal particles.

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17. A use according to Claim ¹⁶18 wherein said patient has developed FVIII inhibitor antibodies.

18. A pharmaceutical composition for parenteral administration comprising a therapeutically effective amount of a protein or polypeptide and substantially neutral colloidal particles, said particles comprising
5 approximately 1-20 mole percent of an amphipathic lipid derivatized with a biocompatible hydrophilic polymer, said polymer carrying substantially no net charge,

wherein said protein or polypeptide is selected from the group
10 consisting of:

- (a) proteins or polypeptides capable of externally binding said colloidal particles; and
- (b) proteins or polypeptides capable of binding polyethylene glycol,
and wherein said protein or polypeptide is not encapsulated in
15 said colloidal particles.

19. Use of a colloidal particle in the preparation of a pharmaceutical composition for parenteral administration comprising a therapeutically effective amount of a protein or polypeptide and substantially neutral colloidal particles, said particles comprising approximately 1-20 mole
20 percent of an amphipathic lipid derivatized with a biocompatible hydrophilic polymer, said polymer carrying substantially no net charge,

wherein said protein or polypeptide is selected from the group
consisting of:

- (a) proteins or polypeptides capable of externally binding said colloidal
25 particles; and
- (b) proteins or polypeptides capable of binding polyethylene glycol,
and wherein said protein or polypeptide is not encapsulated in said colloidal particles.

ABSTRACT

A pharmaceutical composition for parenteral administration comprising a therapeutically effective amount of a protein or polypeptide and substantially neutral colloidal particles. The particles comprise approximately 1-20 mole percent of an amphipathic lipid derivatized with a biocompatible hydrophilic polymer which carries substantially no net charge. The protein or polypeptide is capable of externally binding the colloidal particles, or is capable of binding polyethylene glycol, and is not encapsulated in the colloidal particles. A preferred protein is factor VIII, whose half-life is extended and which is protected from serum inhibitor antibodies by injecting it as a component of the composition.

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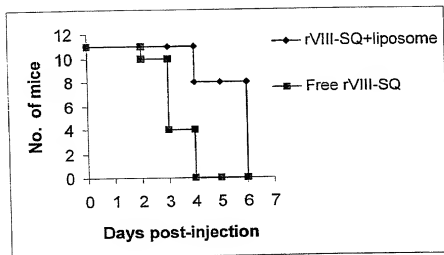


Fig. 1

**DECLARATION AND POWER OF ATTORNEY
UNDER 35 USC §371(c)(4) FOR
PCT APPLICATION FOR UNITED STATES PATENT**

As a below named inventor, I hereby declare that:
my residence, post office address and citizenship are as stated below under my name;

I verify believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought, namely the invention entitled: PHARMACEUTICAL COMPOSITION COMPRISING FACTOR VIII AND NEUTRAL LIPOMES
described and claimed in international application number PCT/IL92/00217 filed April 23, 1992.

I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations §1.56.

Under Title 35, U.S. Code §119, the priority benefits of the following foreign application(s) filed by me or my legal representatives or assigns within one year prior to my international application are hereby claimed:

Israeli Patent Application No. IL 124224 filed April 27, 1998

The following application(s) for patent or inventor's certificate on this invention were filed in countries foreign to the United States of America either (a) more than one year prior to my international application, or (b) before the filing date of the above-named foreign priority application(s):

I hereby appoint the following as my attorneys of record with full power of substitution and revocation to prosecute this application and to transact all business in the Patent Office:

James A. Oliff, Reg. No. 27,075; William P. Berridge, Reg. No. 30,024;
Kirk M. Hudson, Reg. No. 27,562; Thomas J. Partini, Reg. No. 36,411;
Edward P. Walker, Reg. No. 31,450; Robert A. Miller, Reg. No. 32,771;
Mario A. Costantino, Reg. No. 33,566; and Stephen J. Roe, Reg. No. 34,463.

ALL CORRESPONDENCE IN CONNECTION WITH THIS APPLICATION SHOULD BE SENT TO OLIFF & BERRIDGE, P.C., P.O. BOX 19928, ALEXANDRIA, VIRGINIA 22320, TELEPHONE (703) 836-6400.

I hereby declare that I have reviewed and understand the contents of this Declaration, and that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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IF THERE IS MORE THAN ONE INVENTOR USE PAGE 2 AND PLACE AN "X" HERE

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Residence: City State or Province

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Note to Inventor: Please sign name on line 2 exactly as it appears in line 1 and insert the actual date of signing on line 3.

This form may be executed only when attached to the first page of the Declaration and Power of Attorney of the application to which it pertains.